REMARKS

I. Rejections Under 35 U.S.C. § 112, first paragraph (Enablement)

Claims 1-13, 21-26, and 28-30 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. The Office Action alleged that the instant specification and prior art are not enabling for apelin antisense therapy. Applicant respectfully submits that the specification is sufficient to enable one of ordinary skill in the art to make and use the invention as recited in the currently pending claims.

The Office Action of April 19, 2007 acknowledged the working models in the specification for inhibiting vascular growth or angiogenesis in a frog embryo with antisense DNA for apelin (example 5), increased apelin expression in approximately one third of 154 human tumor samples compared to non-tumor tissue (example 6), and upregulation of apelin under hypoxic conditions in primary rat cardiomyocte cells (example 7). The current Office Action also acknowledged the Declaration of Dr. Krieg, dated September 18, 2007, demonstrates that "an apelin antisense oligonucleotide does in fact inhibit angiogenesis" in an art-accepted model. The current Office Action, however, alleges that at the time the instant invention was filed, the art recognized significant unpredictability in the use of antisense molecules. Applicant respectfully submits that the strategy of antisense treatment is sufficiently predictable and, therefore, enabled such that one skilled in the art could practice the claimed invention. For example, the Office Action admits that Chirila teaches that "the antisense strategy only awaits a suitable delivery system in order to live up to its promise." Implicit in that teaching is that antisense strategies are sufficiently developed for predictable application, but this specific author believes the limitation is the delivery system. In contrast, the instant specification provides an *in vivo* model for the use of an apelin antisense oligonucleotide to inhibit angiogenesis. Example 5 shows that an apelin antisense oligonucleotide inhibits angiogenesis in the angiogenesis model system of *Xenopus* embryos. *See* instant specification, pages 33-34, paragraphs [0106]-[0112]. In the present specification, apelin antisense morpholino oligonucleotides (or mismatch controls) were injected into one cell of a 2 cell frog embryo along with Texas Red as a lineage tracer. Embryos were grown to stage 35 and then assayed by *in situ* hybridization using the vascular marker erg. The antisense oligonucleotides resulted in a 67% inhibition of angiogenic growth of

embryonic blood vessels, including inhibition of the development of the intersomitic vessels (*See* Figure 9 of instant specification). Similar results were obtained with antisense oligonucleotides to the APJ receptor; however, mismatch control oligonucleotides did not detectably affect the vascular growth of the embryos. These results show that the apelin antisense molecules specifically inhibit angiogenesis in an art-accepted model system for angiogenesis. Further, the mode of delivery in this example was through injection of the antisense molecules. Therefore the specification demonstrates at least one mode of delivery that successfully administers antisense molecules for modulating angiogenesis. There is no objective evidence in the Office Action that negates this mode of administration or that supports an argument that this mode of delivery is unacceptable. This example, therefore, together with the Chirila reference as cited in the current Office Action, demonstrates that apelin antisense therapy is enabled and one skilled in the art would recognize this therapy is enabled.

The Office Action further alleged that the specification does not demonstrate any working models for an inhibitory apelin antibody. The Office Action noted that a copy of the reference to Kleinz had not been provided to verify the assertion that the antibodies of Kleinz could inhibit apelin activity. Thus, Applicants' response was deemed incomplete. The Applicant respectfully submits that a copy of the reference to Kleinz has now been provided as Exhibit A. Applicants point the Examiner to Figure 2 of Kleinz that discloses the specificity of apelin antibodies to two apelin peptides. This confirms the statement in the Declaration of Kreig, dated September 18, 2007, that states "others have identified additional antibodies that specifically bind apelin." One skilled in the art will recognize that binding of the anti-apelin antibodies to apelin peptides can inhibit apelin activity, block apelin peptide receptor interactions, or block apelin interaction with APJ. Applicant also respectfully submits the Office Action specifically acknowledges the two of the anti-apelin antibodies have anti-apelin activity, which demonstrates that one skilled in the art can readily synthesize anti-apelin antibodies that specifically bind to apelin, and therefore affect the activity of apelin.

The Office Action also alleged that the Applicants have not disclosed which of the apelin epitopes the antibodies ab206, ab 207, ab 208, and ab 210 were made to, thus one could not reasonably conclude that the listed antibodies could bind to any one or more of the peptides of SEQ ID NO: 1-5. Applicant respectfully submits that one skilled in the art would appreciate that

the generation of antibodies is routine in the art as are methods of determining the specificity and effectiveness of those antibodies. Determining those antibodies that bind to and inhibit the activity of apelin is routine. In fact, the specification and the Declaration, together with the relevant skill in the art, enables one to practice the claimed invention because the generation of antibodies to peptides is widely known in the art. The Declaration demonstrates the anti-apelin antibodies can specifically bind to apelin, and thereby affect the activity of apelin in an art-accepted angiogenesis model. Applicant respectfully submit that this enables the claimed invention and it is not required that Applicant demonstrate apelin antibodies that bind to the peptides of SEQ ID NOs:1-5 can generate anti-angiogenesis responses in any sample from any subject, in any species and to what degree. The CAM model is an art-accepted angiogenesis model. The purpose of a model system is to allow one to determine the effectiveness of a treatment in that model to specifically avoid having to perform those tests in any sample from any subject, in any species and to what degree. Applicant respectfully submits that the Office Action appears to reject the validity of the art-accepted models used to demonstrate the claimed invention but does not present any objective evidence why these models are not acceptable.

The Office Action also questioned the relevancy of an anti-apelin antibody binding to a peptide of SEQ ID NO:5 from zebrafish apelin and noted that the Applicants' response did not address this aspect of the rejection and was therefore incomplete. The sequence of SEQ ID NO:5 is virtually identical with SEQ ID NO:4 (with the first base being the only non-identical residue). Therefore, SEQ ID NO:5 should generate similar antibodies relative to the antibodies to SEQ ID NO:4, which is a human-derived protein. Applicant also points out to the Examiner that the 13 C-terminal amino acids on SEQ ID NOs:1-4 are also nearly identical to the sequence of SEQ ID NO:5, with the only change the N-terminal proline residue on SEQ ID NO:5 being a glutamine residue on SEQ ID NOs:1-4, which are all human-derived proteins. Therefore, the specification and the claims are enabled for SEQ ID NO:5, in addition to SEQ ID NOs:1-4.

For at least the foregoing reasons, Applicants respectfully submit that the specification is sufficient to enable one of ordinary skill in the art to make and use the invention as recited in the currently pending claims. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

II. Rejection Under 35 U.S.C. § 112, first paragraph (Written Description)

Claims 6 and 30 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. In particular, the Office Action alleges that the claims recite VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, VEGF-E, PIGF, acidic fibroblast growth factor (FGF-1) for the species of angiogenic factor and that none of those find literal support in the classification. Applicant respectfully submits that prior art literature identifies each of the listed species as angiogenic factors. *See, e.g.*, Felmeden *et al.*, 2003, European Heart Journal 24:586-603; and specifically pages 589-591 for individual descriptions. (Felmeden *et al.* is submitted herewith as Exhibit B) Therefore, it was well known in the art that the angiogenic factors VGF's and FGF's include VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, VEGF-E, PIGF, and acidic fibroblast growth factor (FGF-1). Accordingly, Applicant respectfully requests that the rejections under 35 U.S.C. § 112, first paragraph be withdrawn.

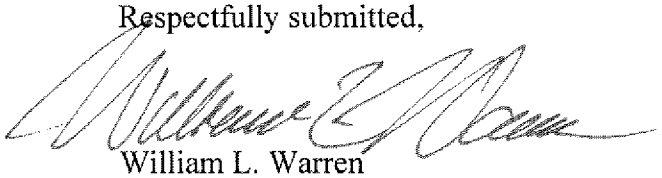
Conclusion

Applicant believes that the present application, as amended, is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The foregoing is submitted as a full and complete response to the Office Action mailed December 6, 2007.

A petition for a three-month extension of time and a request for continuing examination are enclosed, along with the appropriate fees therefor. It is not believed that any additional extensions of time or fees for net addition of claims are required. However, please charge any additional fees that may be due, or credit any overpayment, to Deposit Account 19-5029 (Ref. No.: 20825-0004).

In addition, if there are any issues that can be resolved by a telephone conference or an Examiner's amendment, the Examiner is invited and encouraged to call the undersigned attorney at (404) 853-8000.

Respectfully submitted,



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Date: June 10, 2008

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EXHIBIT A

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Immunocytochemical localization of the endogenous vasoactive peptide apelin to human vascular and endocardial endothelial cells

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Abstract

Apelin, the proposed endogenous peptide ligand of the novel G-protein-coupled receptor APJ, has been shown to possess potent vasodilator and positive inotropic effects in rats and humans in vivo. However, in humans, no endogenous source of apelin has been reported. Therefore, based on the presence of APJ and mRNA encoding apelin in human tissues, we investigated the expression of apelin in fresh-frozen human tissue from right atrium, left ventricle, lung, kidney, adrenal and large conduit vessels using immunocytochemistry. Apelin-like immunoreactivity (apelin-LI) was detected in vascular endothelial cells lining blood vessels in the human heart, kidney, adrenal gland and lung and in endothelial cells of large conduit vessels. Apelin-LI was also present in endocardial endothelial cells lining recesses of the right atrium. Apelin-LI was not present or below the level of detection in cardiomyocytes, Purkinje's cells, pulmonary or renal epithelial cells, secretory cells of the adrenal gland, vascular smooth muscle cells, adipocytes, nerves and connective tissue. The restricted presence of apelin-LI in endothelial cells suggests that endothelial apelin may play a role as a locally secreted cardiovascular mediator acting on APJ receptors present on the vascular smooth muscle and on cardiac myocytes to regulate vascular tone and cardiac contractility.
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Keywords: APJ receptor; Cardiovascular; Endothelium; Orphan G-protein-coupled receptor; Paracrine; Vasodilator

1. Introduction

APJ was first cloned from a human gene by O'Dowd et al. [7] and defined as an "orphan" G-protein-coupled receptor, since the endogenous ligand was not then known. Expression of the receptor in Chinese hamster ovary cells and reverse pharmacology resulted in the isolation of a putative ligand, a 36 amino acid activator peptide from bovine stomach. Consecutive cloning of human and bovine cDNA encoding the novel peptide, which the authors named apelin, led to its identification as the proposed endogenous ligand for the APJ receptor [1]. Interestingly, although apelin-36 was the first natural gene product discovered, testing of shorter synthetic C-terminal apelin peptides (Fig. 1) for activator potential in the same assay showed that shorter fragments were two orders of magnitude more potent than apelin-36, with the N-terminal pyroglutamyl form, (Pyr^1)apelin-13, being most effective [1]. A number of basic amino acids in the sequence of the prepropeptide constitute prerequisite cleaving sites for endopeptidases, and the fact that in bovine colostrum Western blot analysis detected a range of apelin immunoreactive peptides with varying molecular weights suggests the existence of different biologically active apelin peptides [2]. However, the predominant endogenous peptides are (Pyr^1)-apelin-13 and apelin-36. The levels of apelin-36 exceed those of (Pyr^1)apelin-13 in bovine colostrum and rat tissues, but (Pyr^1)apelin-13 is more potent in a number of functional reporter assays. Therefore, evidence suggests that (Pyr^1)apelin-13 is the final product of post-translational modification and the biologically active endogenous ligand [3,4,5,6].

In rat and human brain, apelin and APJ mRNA is abundantly expressed suggesting a central regulatory role for the receptor system [7,8,9,10]. Apelin has also been proposed to be a regulator of fluid homeostasis as the peptide influences water intake in rats, and in the rat hypothalamus apelin is co-localized with vasopressin in neurones of the supraoptic and paraventricular nuclei [6]. In accordance with these findings, other groups have reported changes in water intake as a result of intraperitoneal [10] or intracerebroventricular [6,11] administration of apelin in rats.

In the periphery, we have previously localized apelin receptors in rat and human myocardium as well as in the

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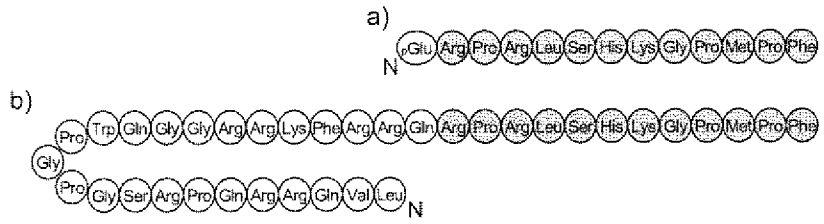

Fig. 1. Amino acid sequences of apelin-13 (a) and apelin-36 (b). Dark grey indicates amino acids identical in both peptides. The N-terminal Pyr^1 of (Pyr^1) apelin-13 differs from the prepropeptide sequence as a result of post-translational modification. The antiserum used for immunocytochemistry was raised against the C-terminal dodecapeptide common to both peptides, which is indicated in dark grey.

medial layer of human coronary artery, aorta and saphenous vein grafts using [^{125}I]-(Pyr^1)Apelin-13 and shown potent constrictor responses to (Pyr^1)Apelin-13 in endothelium denuded, isolated human saphenous vein [12]. In agreement with this receptor distribution, intravenous injection of apelin in anaesthetized and conscious Wistar rats leads to a significant decrease in mean arterial blood pressure [6,10,13,14] and has positive inotropic effects in the isolated rat heart [15], suggesting a role for apelin in cardiovascular regulation. Apelin-like immunoreactivity (apelin-LI) has been reported in endothelial cells of rat blood vessels [13] and apelin mRNA is abundantly expressed in cultured human endothelial cells [16]. Therefore we hypothesise that apelin might be an endothelium derived vasoactive mediator, however, the cellular distribution of the peptide in human tissue, has not been examined. We have investigated the presence of apelin-LI in fresh-frozen human tissue using an antiserum raised against the C-terminal dodecapeptide of the apelin sequence.

2. Materials and methods

2.1. Materials

Unless stated, all chemicals were obtained from Sigma-Aldrich (Poole, UK). Rabbit-anti-apelin-12 and rabbit-anti-apelin-36 antiserum used in immunocytochemistry was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Mouse-anti-human von Willebrand factor monoclonal antibody, secondary antibodies, rabbit-PAP-complex and horseradish-peroxidase-conjugated swine-anti-rabbit antiserum were from DAKO (Glostrup, Denmark). The 96-well microtiter plates were from NUNC (Roskilde, Denmark) and DePeX-Gurr mounting medium from BDH Laboratory Supplies (Poole, UK).

2.2. Tissue collection

Human tissues were obtained with local ethical approval. Left ventricular and atrial myocardium were taken from patients undergoing heart transplants for ischaemic heart disease ($n=6$) or cardiomyopathies ($n=5$), heart lung transplants for cystic fibrosis ($n=3$), or from donor hearts for which there was no suitable recipient ($n=2$). Saphenous veins ($n=12$), radial arteries ($n=4$) and left internal mammary arteries ($n=10$) were collected from patients undergoing coronary artery bypass graft surgery for ischaemic heart disease. Coronary arteries were obtained from patients undergoing heart transplants for ischaemic heart disease ($n=9$), dilated cardiomyopathy ($n=2$) and donor hearts not required for further transplantation ($n=2$). Histologically normal kidney ($n=5$) and lung ($n=5$) was from patients undergoing nephrectomy and lobectomy, respectively, for non-obstructive carcinomas. The histologically normal adrenal tissue was obtained from two patients undergoing adrenalectomy for phaeochromocytoma. On collection, tissues were snap frozen in liquid nitrogen and stored at -70 °C until required.

2.3. Specificity control ELISA

To confirm the specificity of the antiserum interaction with apelin peptides, an ELISA comparing antibody–antigen interaction of the rabbit-anti-apelin-12 serum with ape-

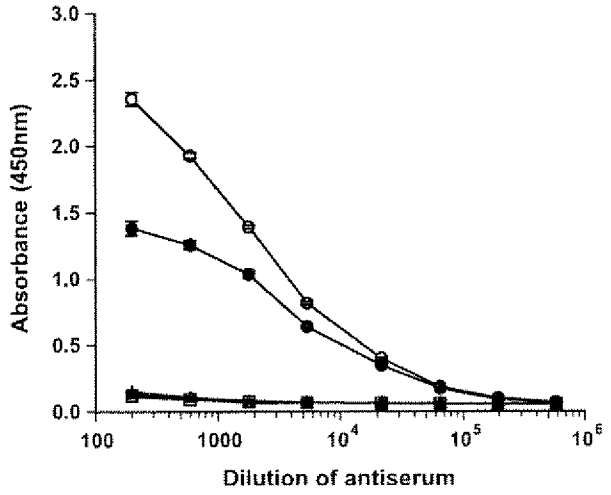

Fig. 2. Specificity of the rabbit-anti-apelin-12 antiserum determined by antiserum dilution ELISA. Microtiter plates were coated with a fixed amount (1 μg/ml) of five different peptides, apelin-36 (○), apelin-13 (●), ghrelin (☆), ET-1 (△), angiotensin-II (□). The antiserum cross-reacted with apelin-36 and apelin-13, but not with the other peptides. Absorbance (mean ± S.E.M.) is plotted against antiserum dilution as a measure of potency and specificity of the antibody–antigen interaction.

lin-13, apelin-36 and three control peptides was designed. As control peptides we chose endothelin-1 and ghrelin, which have both been shown to be present in the vasculature, and angiotensin-II because of its affinity to the APJ-homologous AT-1 receptor. 96 well plates were adsorbed either with apelin-13, apelin-36 or the three control peptides by overnight incubation at 4 °C with 100 μl of a 1 μg peptide/ml solution per well. The plates were washed with phosphate

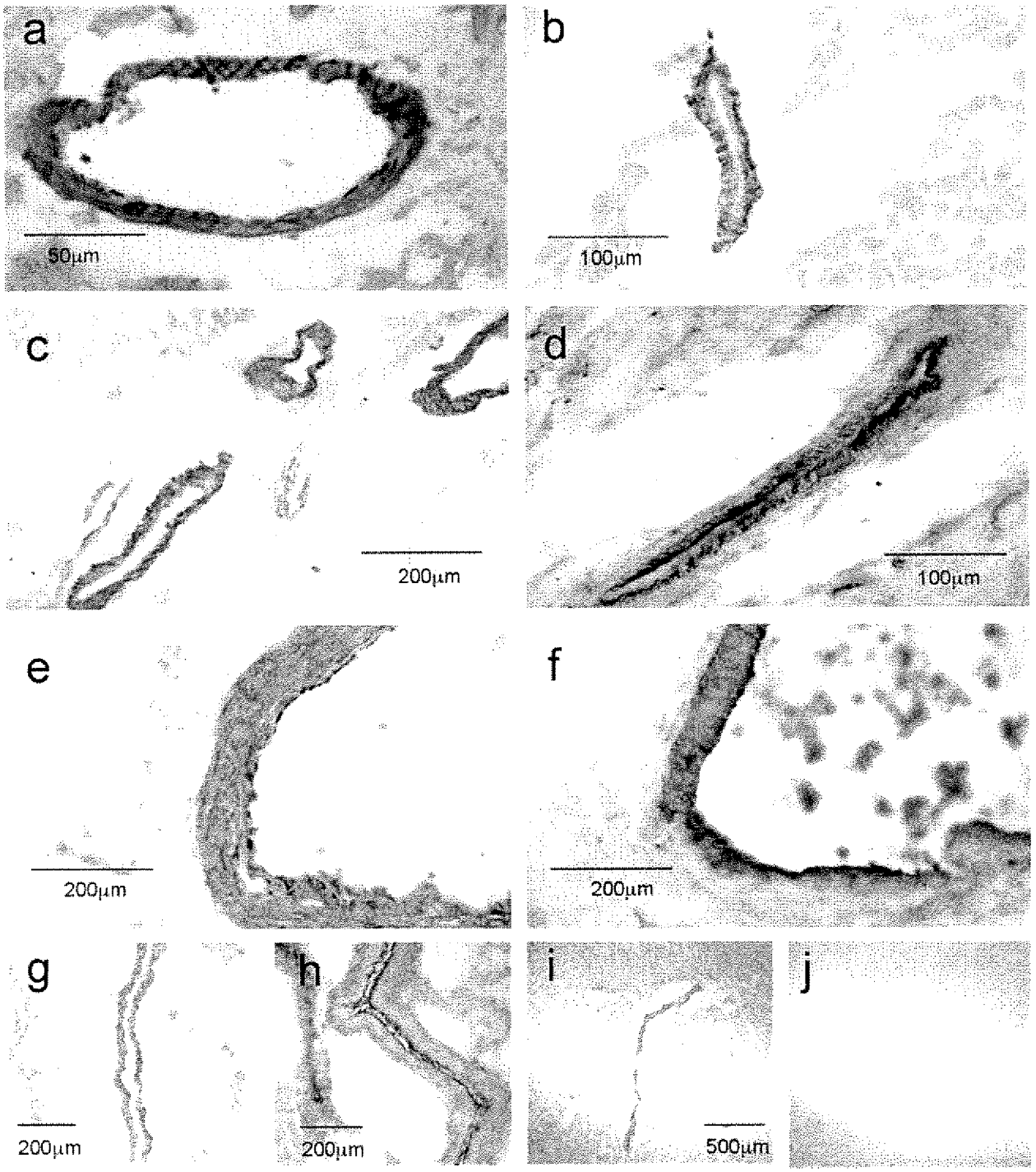

Fig. 3. Photomicrographs demonstrating apelin-like immunoreactivity (apelin-LI) in the human heart. Apelin-LI is expressed in vascular endothelial cells of right atria (a and b) and left ventricle (c and e), with adjacent sections (d and f) stained for the endothelial marker von Willebrand factor (vWF). Apelin-LI was also identified in endocardial endothelial cells lining the right atrium (g) with an adjacent section stained for vWF (h). In sections from right atrium (i) no staining was detectable when the primary antiserum was omitted as negative control (j).

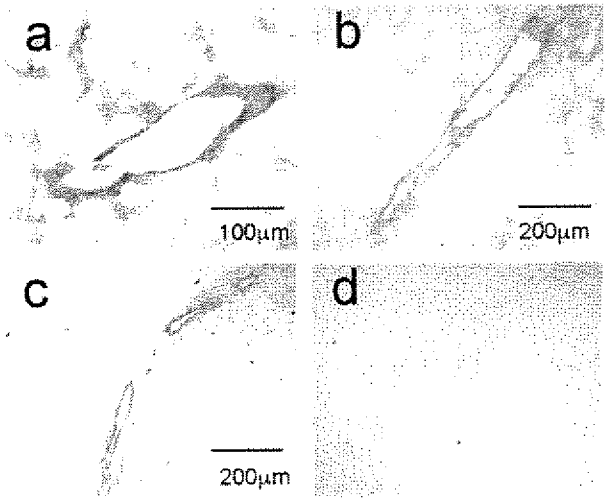

Fig. 4. Photomicrographs showing apelin-like immunoreactivity in vascular endothelial cells of blood vessels from kidney (a, b) and adrenal gland (c) with an adjacent section of adrenal gland (d), where the primary antiserum was omitted as negative control.

buffered saline (PBS) containing 0.1% Tween-20 (PBS/T) and incubated with 3% bovine serum albumin (BSA) in PBS (400 μl/well) for 2 h to block non-specific protein interaction. After repeated washing, wells were incubated overnight with decreasing concentrations of rabbit-anti-apelin-12 antiserum (1:200–1:437400) at 4 °C. Further washing preceded a 2 h incubation with 100 μl of horseradish-peroxidase-conjugated swine-anti-rabbit antiserum at a dilution of 1:2000 in PBS/T containing 1% BSA. Following a further washing step, 100 μl of 3,3′,5,5′ Tetramethylbenzidine chromogenic substrate (TMB) was added to each well to visualize the antibody–antigen complex. After transformation of the blue colour complex using 100 μl 1M H_2SO_4, plates were read at 450 nM. ELISA was carried out in triplicate for all peptides, and the antibody–antigen interaction measured as absorbance (mean ± S.E.M.) plotted against antiserum dilution using the non-iterative curve-fitting programme FigP 2.8 (Biosoft, Cambridge, UK).

2.4. Immunocytochemistry

Cryostat cut tissue sections (10 μm for atrial and ventricular myocardium, lung, kidney and adrenal gland. 30 μm for saphenous vein, radial artery, left internal mammary artery and coronary artery) were left to dry overnight at room temperature. Tissue was fixed in ice-cold acetone for 10 min. Sections were incubated with 5% non-immunized swine serum in PBS for 1 h at room temperature to block non-specific protein interaction. Tissues were incubated with rabbit-anti-apelin-12 (human/rat) antiserum at a dilution of 1:500 in PBS/T containing 1% non-immunized swine serum (1%SS PBS/T) for 72 h at 4 °C. Adjacent sections were stained using rabbit-anti-apelin-36 (human) antiserum at a dilution of 1:200 in PBS/T for 24 h at 4 °C. In adjacent sections, primary antisera were omitted as a negative control or tissue was stained for the endothelial marker von Willebrand factor to test for the presence of endothelial cells. Von Willebrand factor primary antibody was used at 1:1000 dilution for 1 h at room temperature. Tissue sections were then washed three times for 5 min in cold PBS/T before the incubation with swine-anti-rabbit antiserum at a dilution of 1:200 in 1%SS PBS/T for 1 h at room temperature. After repeated washing, sections were incubated with rabbit peroxidase/anti-peroxidase complex at a 1:400 dilution in 1%SS PBS/T. Further washing preceded a 3-min incubation of tissue sections with a 2,5% solution of 3,3′ -diaminobenzidine in 0.05M Tris–HCl buffer containing 0.3% hydrogen peroxide. The chromogenic reaction was stopped by immersing the slides in distilled water. Sections were dehydrated using a graded alcohol series before being submerged in xylene for 1 h to clear. Sections were mounted using DePeX-Gurr mounting medium and then examined using a standard light field microscope (Reichert-Jung, Austria).

2.5. Haematoxylin–eosin staining

Sections adjacent to the sections used in immunocytochemistry were stained using the haematoxylin–eosin method to assess the morphology of the tissues used for immunocytochemistry.

3. Results

3.1. Specificity control ELISA

In the ELISA, the rabbit-anti-apelin-12 serum used in our immunocytochemistry experiments potently and specifically detected apelin-13 and apelin-36 resulting in a strong signal,

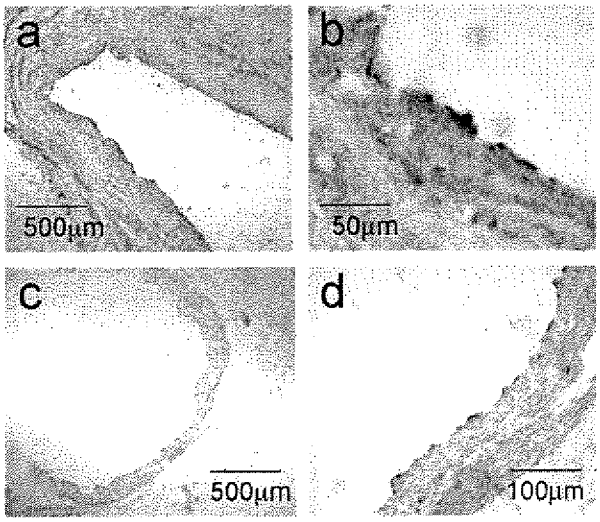

Fig. 5. Apelin-like immunoreactivity in vascular endothelial cells of large conduit vessels. Photomicrographs show staining in saphenous vein (a) and coronary artery (c) with higher magnification of the respective vessels in (b) and (d).

even at greater dilutions than those used in the immunocytochemistry protocol. The antiserum bound the apelin-36 fragment with ~ 60% higher potency compared to apelin-13 at the concentration range used in the immunocytochemistry protocol. The antiserum did not detect the three endothelial peptides angiotensin II, endothelin-1 and ghrelin (< 5% cross reactivity) (Fig. 2).

3.2. Immunocytochemistry

In sections of human atrial and ventricular myocardium we detected apelin-LI in endothelial cells lining small intramyocardial blood vessels, small coronary arteries and in endocardial endothelial cells lining the recesses of the atrial chamber (Fig. 3). In cardiomyocytes, Purkinje's cells, vascular smooth muscle cells and coronary adipocytes apelin-LI was absent or below the level of detection. In the lung, apelin-LI was restricted to endothelial cells of small pulmonary vessels. We did not detect apelin-LI in vascular smooth muscle of pulmonary vessels, pulmonary epithelium or connective tissue. In sections from kidney apelin-LI was present in endothelial cells lining small intrarenal vessels (Fig. 4). No staining was detected in vascular smooth muscle cells of the respective vessels, glomeruli, renal tubular epithelial cells or connective tissue. In the adrenal gland, apelin-LI was confined to endothelial cells of the surrounding arteries, small resistance arteries within the capsular plexus and the central vein (Fig. 4). In secretory cells of both the adrenal cortex (zona glomerulosa, zona fasciculata, zona reticularis) and medulla apelin-LI was not detectable. In human large conduit vessels we observed apelin-LI in endothelial cells lining the walls of saphenous veins, coronary arteries and left internal mammary arteries (Fig. 5) but not in the vascular smooth muscle cells forming the vessel wall. In adjacent sections apelin-36-like immunoreactivity (apelin-36-LI) was not present or below the level of detection. Staining was absent in sections where the primary antiserum was omitted as a negative control. Staining for human von Willebrand factor detected endothelial cells in sections used as a positive control for the presence of endothelium.

4. Discussion

This is the first report of widespread presence of apelin-LI in vascular endothelial cells of fresh-frozen human tissues. The peptides detected by our immunocytochemistry protocol represent the complete range of known apelin peptides, as the primary antiserum has been raised against a C-terminal dodecapeptide from the preproapelin sequence, a sequence common to all functionally active apelin fragments examined so far. Additionally our enzyme-linked immunosorbent assay demonstrated that the antiserum detects short and long apelin peptides and shows no cross reactivity with unrelated peptides. However, studies showing predominant expression of (Pyr^1)apelin-13 and apelin-36 in rat and bovine tissue and colostrum [3,4] and prominent functional potency of (Pyr^1)apelin-13 compared to other apelin fragments, lead us to hypothesise that the majority of peptides present in the tissue is the mature ligand (Pyr^1)apelin-13 [1]. This hypothesis is supported by the fact that apelin-36-LI was not detected in adjacent sections.

Our findings are in accordance with studies showing apelin-like immunoreactivity in vascular endothelial cells in rats [13] and the discovery of significantly higher levels of apelin mRNA in human cultured vascular endothelial cells compared to vascular smooth muscle [16]. Moreover, the restricted expression of apelin peptides to vascular endothelial cells that we report provides a possible explanation for the low levels of expression of mRNA encoding apelin throughout rat tissues and the high levels of expression in lung and mammary gland using RT-PCR [2,4,16]. Consistent with endothelial cells lining all vessels in all organs, low levels of apelin mRNA are present in all tissues, whilst in highly vascular parenchyma such as lung and mammary gland apelin mRNA levels are significantly higher.

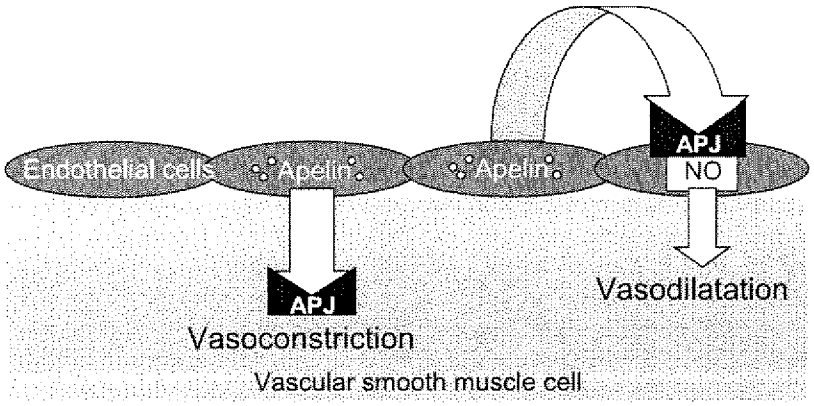

Fig. 6. Schematic summary of proposed vascular functions of apelin.

Our report of the abundant distribution of apelin-LI in vascular endothelial cells provides an essential clue to understand the functional roles of apelin in the regulation of the cardiovascular system. In vivo studies investigating the effect of intravenous administration of apelin peptides in rats so far consistently report a significant decrease in mean arterial blood pressure [6,10,13,14]. Tatemoto et al. [13] showed that this hypotensive effect is negatively correlated with peptide size (apelin-12>apelin-13>apelin-36) and that it can be abolished by co-administration of the nitric-oxide synthase inhibitor L-NAME. In the light of these functional observations the importance of the discovery of apelin-12 LI in vascular endothelial cells is emphasised, as so far in man no endogenous peripheral source of the peptide was known. We therefore propose a role for apelin as a local vasoactive mediator. The peptide, produced and released from vascular endothelial cells, may activate APJ receptors present in close proximity on the underlying vascular smooth muscle to elicit its vascular functions. The paracrine nature of apelin function (Fig. 6) may explain why the ligand of this endogenous receptor system has so far not been detected in the circulation in significant amounts [4].

The postulate that exogenous apelin acts through an endothelium dependent mechanism [13] is further supported by previous findings made by our group. In endothelium denuded isolated human saphenous veins apelin is a potent vasoconstrictor with nanomolar potency and a maximum response comparable to that of angiotensin II [12]. This opposing effect of apelin in endothelium-denuded vessels implies that the direct action of endothelial apelin on vascular smooth muscle is vasoconstriction rather than dilation—at least in this type of vessel. The fact that the vasodilation after systemic administration of apelin is abolished by L-NAME suggests the additional presence of an endothelium dependent mechanism mediating the vasodilator action. Thus, in conjunction with our immunocytochemistry results we propose that endothelial apelin, acting on vascular smooth muscle in a paracrine fashion has vasoconstrictor potential. However, in the presence of a functional endothelium this vasoconstrictor effect may be counterbalanced or even masked by autocrine activation of APJ receptors on vascular endothelial cells resulting in the release of endothelial vasodilator substances, such as nitric oxide (Fig. 6). The concept of a pathway signalling via endothelial APJ is supported by a report of msr/apj expression, the mouse analogue of human APJ, in vascular and endocardial endothelial cells of mouse embryo [17]. In the heart, apelin may have a similar role as a local mediator, since we detected apelin-LI in endocardial endothelial cells lining atrial recesses.

Positive inotropic actions for apelin in isolated rat heart have been reported, suggesting a regulatory role for apelin in cardiac contractility [15]. The endocardial endothelium, as a part of the cardiac wall, is the first anatomical structure in the heart exposed to pressure changes due to variation of cardiac activity or marked changes in the peripheral circulation. Endothelial cells are extremely sensitive to shear stress, which can trigger the liberation of endothelial factors from the cells, as demonstrated for endothelin-1 [18] and matrix metalloproteinase (MMP)-2 [19]. Hence apelin, which we have shown to be present in endocardial endothelial cells, might be released from these cells as an endothelial response to increased cardiac pre- or afterload to improve cardiac contractility. In turn, a lack of apelin could contribute to the development of dilated cardiomyopathy. In conclusion, we have demonstrated the widespread localization of apelin-12 LI in endothelial cells of the human vasculature and endocardial endothelial cells and propose a role for the apelin/APJ receptor system as a regulator of vascular tone and cardiac contractility.

Acknowledgements

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References

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EXHIBIT B

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Review article

Angiogenesis: basic pathophysiology and implications for disease

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Introduction

The development of new blood vessels is essential to embryonic growth and throughout life for physiological repair processes such as wound healing, post-ischaemic tissue restoration, and the endometrial changes of the menstrual cycle. However, abnormal development of new blood vessels has been implicated in numerous pathophysiological processes. For example, inhibited growth of blood vessels is associated with bowel atresia and peptic ulcers.[1–3] Furthermore, although generally focussing on tumour growth, increased vascular growth has been demonstrated in many other non-malignant diseases such rheumatoid arthritis, systemic lupus erythematosus, psoriasis, proliferative retinopathy and atherosclerosis.[3–5] It is therefore clear that the subject is currently attracting considerable research energies as tools are becoming available to assess possible therapeutic options.

The formation of the vascular system is fashioned by three processes. During embryogenesis, there is differentiation of embryonic mesenchymal cells (the endothelial precursor cells or angioblasts) into endothelial cells resulting in de novo development of blood vessels (vasculogenesis).[6] Secondly, angiogenesis refers to the formation of new blood vessels by sprouting from pre-existing small vessels in adult and embryonic tissue (sprouting angiogenesis) or by intravascular subdivision (intussusception). The existing vasculature can be transformed into a mature network by processes of pruning and remodelling. Thirdly, arteriogenesis is defined as rapid proliferation of pre-existing collateral vessels.[7] Angiogenesis also seems to be an organ-specific process reliant on the stage of microvascular network.[8]

Since angiogenesis seems to play a key role in the pathophysiology of various disease processes, recent attempts have been made to utilize this

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knowledge in the development of new therapeutic approaches. For example, inhibition of angiogenesis has been used in the restriction of tumour growth and the seeding of metastases, as well as in rheumatoid arthritis, where an aim is to reduce the infiltration of inflammatory cells and soluble mediators.[9–11]

Angiogenesis related research in cardiovascular medicine has initially been linked to ischaemic heart disease and atherosclerosis. The observed raised angiogenic markers resulted in a theory of impaired angiogenesis in cardiovascular disease.[12] One therapeutic direction in ischaemic vascular disease has been to use various angiogenic growth factors in an effort to improve vascularization,[12–14] and more recently the role of angiogenesis in hypertension has also been investigated.[15] However, in order to discuss the potential implications of angiogenesis in disease states, the mechanisms of vascular growth need to be fully understood.

Search strategy

In order to achieve our objective of summarizing current literature on angiogenesis, fibroblast growth factor (FGF) and vascular endothelial cell growth factor, we entered these and other key words into online literature search engines such as PubMed and EMBASE, as well as obtaining data and copy from other current reviews, reference lists of current literature, information from expert colleagues and abstracts from meetings of relevant societies.

Basic mechanisms of blood vessel formation

Vasculogenesis

In embryogenesis, vasculogenesis is a complex but ordered process involving the differentiation of endothelial precursor cells (angioblasts) from primitive mesoderm commencing with gastrulation.[16,17] This process is probably induced by FGF.[18] The angioblasts can be distinguished adjacent to primitive blood cells, and are located in distinct zones that when merged together are the first indication of a primitive vasculature. In the next step, these mesoderm-derived angioblasts differentiate into endothelial cells and form de novo vessels.[19] The process of vasculogenesis occurs predominantly during embryonic development. These initial blood vessels consist purely of endothelial cells and are referred to as capillary plexus.[8] The succeeding development of various diverse blood vessels is a complex process. The ultimate vessel structure is determined by the derivation of the endothelial cells and smooth muscle cells comprising the vessel wall.

The process of subendothelial smooth muscle cell layer development incorporates migration, and proliferation of different cell types such as pericytes, smooth muscle cells and fibroblasts. The precise mechanisms involved in early vessel formation have yet to be elucidated but observations indicate that the primordial endothelium can recruit undifferentiated locally derived mesenchymal cells and direct their differentiation into pericytes in microvessels, and smooth muscle cells in large vessels.[20] In comparison to the rather uniform endothelial cells, vascular smooth muscle cells are much more diverse. They can develop from endothelial cells as well as fibroblasts.[21,22] In addition to endothelial and splanchnic mesodermal origin, there is also evidence of derivation from the mesectoderm of the neural crest.[23,24] The diverse origin of the vascular smooth muscle cell is an important factor in the tissue specific make-up of the final blood vessel.

During vasculogenesis, mesodermal precursor cells form a primitive vascular plexus. Vascular structures such as the dorsal aorta and the heart are also formed. This process involves the differentiation and organization of endothelial cells into capillary tubes and the interplay between growth factors and cytokines. The subsequent process of remodelling of the primary capillary plexus is termed angiogenesis.[25]

Embryonic angiogenesis

The primary step of angiogenesis is thought to be initiated by activation of endothelial cells of pre-existing vessels in response to increasing levels of local angiogenic stimuli. This results in local vasodilatation, increased vascular permeability and the disruption of the basement membrane encompassing endothelial cells of the existing capillaries via proteolytic degradation.[26] These enzymes may be activated by growth regulatory molecules.[27] The disturbance of the basement membrane allows cytoplasmatic processes to extend from the activated endothelial cells, directing their migration and sprouting into the extravascular space toward the angiogenic stimulus. After the proliferation, elongation and alignment of the endothelial cells follows the formation of capillary sprouts. The growing sprout eventually develops a lumen and consequently these tubular structures anastomose with neighbouring vessels. The resulting capillary

Table 1 Key events of angiogenesis

Phase	Key events
Endothelial cell and pericyte activation	Morphological changes of endothelial cells priming them for proliferation and secretion, local vasodilatation, increased vascular permeability, accumulation of extravascular fibrin
Degradation of basement membrane	Angiogenic stimulus results in proteolytic vascular basement membrane degradation
Migration of endothelial cells	Chemotactic factors produced by fibroblasts, monocytes and platelets induce endothelial cell migration and sprouting
Proliferation of endothelial cells	Locally produced mitogens induce endothelial cells DNA synthesis and mitosis
Differentiation of endothelial cells	Endothelial cell proliferation decreases and cell–cell contact re-establish, sprout develops lumen
Reconstitution of basement membrane	Vessel maturation achieved by reconstitution of basement membrane synthesized by endothelial cells and pericytes
Vasculature maturation and stabilization	Capillary remodelling by stabilization and regression

From references [6,8,29,30] and elsewhere.

loop then permits blood flow.[8,25] In the final stage these vessels are again remodelled by stabilization and regression. The development of establishing and remodelling of blood vessels is believed to be mediated by paracrine signals, and the formation of the basement membrane completes the maturation process.[28–30]

Post-embryonic angiogenesis

In post-embryonic development the main form of vasculature expansion is angiogenesis, also referred to as neovascularization. Post-embryonic angiogenesis follows the pattern of embryonic angiogenesis, and as tissue grows expansion of the vasculature is essential. This process includes growth and disappearance of capillaries and formation of arterioles and venules[6,8,28] (Table 1). Angiogenesis also involves the differentiation and organization of endothelial cells into capillary tubes and the interplay between growth factors and cytokines. Cell adhesion molecules generally mediate innumerable cell–cell and cell–matrix interactions. These, in conjunction with the recruitment of supporting pre-endothelial cells that encase the endothelial tubes, provide maintenance and modulatory functions to the vessel. Supporting cells usually include pericytes in small capillaries and smooth muscle cells in larger vessels.[29,30]

In a healthy mature organism endothelial cell turnover is, with the exception of angiogenesis, very low. Angiogenesis is essential during vessel growth in most organs particularly in pathophysiological processes occurring in response to injury such as gastrointestinal ulcers, strokes, myocardial infarction and left ventricular hypertrophy.[31–34] Female reproductive organs demonstrate ongoing physiological angiogenesis to ensure the proper biological functioning of these organs during their lifespan.[35–37] The expression of numerous angiogenic growth factors is required in the development of ovarian follicles and corpus luteum.[38,39]

Angiogenic growth factors

The existence of angiogenic factors was first observed with the isolation of a tumour factor that generated mitogenic activities in endothelial cells and later found to be a member of the FGF family.[40] Angiogenetic growth factors are produced by a variety of different cells, and their functions include close involvement in developmental as well as tumour angiogenesis.[41] Indeed, angiogenic growth factors such as vascular endothelial growth factor (VEGF), FGF and angiopoietin are essential to angiogenesis.[19,40–42] Further to the initiation of angiogenesis these growth regulators establish the rate and extent of angiogenesis. However, little data are available about the resolution phase of angiogenesis. It is still unclear if this process results from exhaustion of the growth factors or if negative regulators predominate in this phase.

Angiogenic growth factors are so-called because of their varying ability to induce the proliferation of various cells in vitro, which contribute to the process of angiogenesis in vivo, as demonstrated by studies of animal models (Table 2). These growth factors are produced by various cell types and include a diverse range of proteins in addition to VEGF and FGF: platelet derived growth factor, tumour necrosis factor, insulin like growth factor-1, transforming growth factor, angiogenin, hepatocyte growth factor, placental growth factor and several others.[43,44] Of the vast number of angiogenetic growth factors described, the FGF and VEGF families have been most extensively researched and will be described in more detail.

Table 2 Phenotypes of transgenic mice with embryonic defects in vascular development

Affected gene	Stage of vessel development	Detected phenotype
VEGF-A	Vasculogenesis and angiogenesis	Malformation of dorsal aorta, defective heart and vessel sprouting. Delayed EC differentiation
VEGFR-1	Vasculogenesis	Disordered EC assembly causing enlarged blood vessel and impaired vasculogenesis
VEGFR-2	Vasculogenesis	Undifferentiated EC result in anomalous vessel structure and breakdown of vasculogenesis
VEGFR-3	Vasculogenesis	Abnormal vessel sprouting, organization and remodelling
Angl	Angiogenesis	Impaired neural tube angiogenesis. Deficient vascular remodelling and endocardial branching
Ang2	Maturity	Deficient vessel integrity leading to haemorrhage and vascular oedema
Tie-1	Maturity	Deficient vessel integrity leading to haemorrhage and vascular oedema
Tie-2	Angiogenesis	Defective vascular remodelling and endocardial branching. Impaired neural tube angiogenesis
Neu-1	Angiogenesis	Inadequate development of vascular networks

Modified from references [190,191] and elsewhere.

Fibroblast growth factor

The first angiogenic growth factor to be discovered,[40] this family currently comprises at least 20 molecules with extensive mitogenic potentials representing some of the most potent angiogenic peptides. They are produced by vascular endothelial and smooth muscle cells, hence their almost omnipresent distribution. With numerous biological activities, including induction of proliferation of a wide range of cells, the FGFs are closely involved in several developmental and pathophysiological processes.[44,45] They stimulate fibroblast as well as endothelial cell growth and are therefore of vital importance in the process of angiogenesis,[41] and also play a significant part in at least three of the four phases of wound healing: inflammation, repair and regeneration.[42,46,47] Further important functions of FGFs include tumour development and progression.

One characteristic of the FGF family is the ability to interact with heparan-like glycosaminoglycans of the extra-cellular matrix.[48] The biological responses of FGF are mediated through the activation of four specific receptors, membrane-spanning tyrosine kinases resulting in an increase of multiple isoforms of FGF due to alternative mRNA splicing.[45,49] The two most widely researched isoforms are FGF-1 and FGF-2.

Fibroblastic growth factor-1

Also known as the acidic FGF, in its mature form it is a 16 kD peptide. FGF-1 (as well as FGF-2) does not have a signal peptide for channelling through the classical secretory pathway, but possesses a nuclear localization motif.[50,51] FGF-1 has also been shown to stimulate DNA synthesis without signalling through a cell surface receptor, suggestive of an intracrine mechanism transmitting a nuclear localization signal.[52]

Like other members of the family, FGF-1 has mitogenic and chemotactic effects especially on fibroblasts, endothelial cells and smooth muscle cells. It also contributes to the control of capillary progression, wound healing and tumour progression. Not surprisingly, FGF-1 expression is increased during regeneration of endothelial cells, hypoxia and collateral formation.[44,53–55] However, so far in vivo studies looking into its potential therapeutic use have been disappointing.[56]

Fibroblastic growth factor-2

This single chain 18 kDa polypeptide is also referred to as basic FGF and has a 55% sequence identity with FGF-1.[57] Hypoxia, in addition to a number of other growth factors, increases its activity.[55] FGF-2 is one of the most potent mitogens and chemotactic factors of the vascular endothelial cell. Recently, it has been demonstrated that basic FGF and VEGF have synergistic effects on angiogenesis in vivo.[58] Numerous studies are currently investigating the potential role of FGF-2 and VEGF in the treatment of coronary artery disease.

Vascular endothelial growth factor

Initially purified as vascular permeability factor (VPF) from tumour cell ascites,[59] its biological effects were subsequently shown to extend to endothelial cell mitogenesis, prompting the name change to VEGF.[60–62]

VEGF is now known to be a multifunctional peptide capable of inducing receptor-mediated endothelial cell proliferation and angiogenesis both in

Table 3 Properties of the members of the VEGF family

VEGF protein (references in superscript)	Chromosomal location	Soluble VEGF isoform	Heparin-binding	Heparan-sulphate proteoglycan binding isoform
VEGF (VEGF-A)[70,72]	6q21.3	VEGF-A_{165}, VEGF A_{145}, VEGF-A_{121}	VEGF-A_{189}, VEGF-A_{206}, weakly: VEGF-A_{121}, VEGF A_{145}, VEGF-A_{165}	VEGF-A_{145}, VEGF-A_{189}, VEGF-A_{206}
VEGF-B[81,82]	11q13	VEGF-B_{167}	VEGF-B_{167}	VEGF-B_{189}
VEGF-C[66,85]	4q34	No		No
VEGF-D[92]	Xq22.31	Yes	Yes	No
VEGF-E[97]	Orf Virus genome	Yes	No	No
PlGF[100]	14q24	PlGF-1	PlGF-2, PlGF-3	PlGF-1

vivo and in vitro.[60–63] In addition to its crucial role in embryonic vascular development, VEGF has been implicated in the process of neovascularization in adult pathophysiology.[63–65] VEGF is a basic, 45 kDa disulfide-linked dimeric glycoprotein, that binds heparin and is structurally related to platelet derived growth factors.[63] VEGF loses all biological activities following reduction and dissociates into monomeric units between 17 and 23 kDa.[60] The various VEGF iso-proteins have been described which have a circulating half-life of between 10 min and 6 h, depending upon the isoform, and the exogenous stimulus.[66–69] The whole VEGF family currently consists of at least five members whose effects are mediated via three VEGF receptors (VEGFR), (Table 3). These receptors communicate with the cell interior via transmembrane receptor tyrosine kinases (RTKs).

VEGF-A (VEGF)

Interestingly, human chromosome 6p21.3, that encodes for the VEGF-A gene, the first VEGF protein identified, is also a location giving origin to several human disorders with unidentified genetic defects.[70,71] The VEGF gene sequence extends over approximately 14 kb, encoding eight exons that are separated by seven introns.[72,73] Through alternate exon splicing of this gene different mRNA are encoded producing five biologically active proteins ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$).[62,72–74] All VEGF-A transcriptions have the amino terminal 141 amino acids in common. This consists of a signal peptide enabling its identification by VEGFR Flt-1 and KDR. Exons six and seven code for peptides determining the capability of binding to the extra-cellular matrix and/or heparan sulphate proteoglycan. All VEGF isoforms are secreted glycoproteins. They are able to homodimerize and bind to heparin (except $VEGF_{121}$).[75,76]

$VEGF_{165}$, often referred to as VEGF-A or simply VEGF, is the predominant human isoform secreted by a variety of normal and transformed cells. Although all human VEGF-A isoforms are able to induce in vivo angiogenesis,[73] there are, however, differences in their capability to bind heparan sulphate and VEGFR (Flt-1). The soluble glycoproteins $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ can be detected by biochemical assays (e.g. ELISA) of fluid samples such as human serum and plasma.[77–80] $VEGF_{121}$ is a weakly acidic polypeptide failing to bind to heparan sulphate, whereas the VEGF isoforms $VEGF_{189}$ and $VEGF_{206}$ are more basic and exhibit higher affinity to heparin than $VEGF_{165}$.[72] The differences in the affinity for heparan sulphate and in the isoelectric point have a profound effect on the bioavailability of VEGF, leaving larger VEGF isoforms almost completely cell associated and bound to extra-cellular matrix.[74,75] Only the isoform $VEGF_{165}$ is freely diffusible and able to bind to heparin, which is an indicator of its mitotic activity for vascular endothelial cells. There is also evidence to suggest that the stability of the VEGF–heparan sulphate receptor complex may contribute to effective signal transduction and therefore proliferation of the vascular endothelial cells. In contrast, $VEGF_{206}$ is the rarest isoform and has so far only been discovered in human foetal liver cDNA library.[74–76]

VEGF-B

This member of the VEGF gene family is composed of 188 amino acids and can be expressed as homodimer or heterodimer with VEGF-A..[81–83] Alternate splicing of the VEGF-B gene, situated on chromosome 11q13, results in two isoforms. VEGF-B_{167} is a soluble peptide and VEGF-B_{189} is bound to the cell and extra-cellular matrix[82] and has been shown to stimulate vascular endothelial cell proliferation. These findings resulted in the hypothesis that VEGF-B may contribute to the regulation of angiogenesis in muscle tissue.[81]

VEGF-C

VEGF-C is a protein composed of 419 amino acids, with a predicted molecular mass of 47 kDa whose gene is located on chromosome 4q34.[83,84] VEGF-C shares 30% of the VEGF homology domain and can be found in small quantities in myocardium, placental tissue, skeletal muscle, ovaries, in certain tumour cell lines and is present in platelets.[66,85,86] It is involved in the formation and maintenance of the venous and lymphatic systems and promotes lymphatic endothelial cell proliferation and vessel enlargement.[87–89] Nonetheless, there is also data to suggest that VEGF-C may possess angiogenic properties relating to capillaries.[90] The actions of both VEGF-C and VEGF-B are mediated via their receptors Flt-1 and Flt-4 resulting in a paracrine pathway.[85,91]

VEGF-D

The latest member of the human VEGF family to be described in detail, VEGF-D, shares 61% homology with VEGF-C and its gene is located on chromosome Xp22.31.[92] Human VEGF-D seems to be generated by proteolytic processing of precursor polypeptides.[93,94] VEGF-D is recognized by VEGFR-2 and VEGFR-3, which are present on endothelial cells,[93] and appears to be capable of stimulating lymphangiogenesis.[95] There is further evidence to suggest that VEGF-D may promote the spread of tumour cells via the lymphatic system.[96]

VEGF-E

Based on the sequence of VEGF-A_{121}, a further VEGF variant, VEGF-E, was discovered in the genome of Orf virus.[97] The Orf virus is an epitheliotropic parapoxvirus which induces proliferative skin lesions in goats, sheep and humans (seen as 'milker's nodules').[98] In addition to the characteristic cysteine residue present in all mammalian VEGF proteins, VEGF-E possesses a conserved threonine and proline rich region at the carboxyl terminus.[97] VEGF-E binds with high affinity to VEGFR-2 resulting in stimulation of angiogenesis and vascular permeability, therefore enhancing viral infection.[99]

Placenta growth factor

The first VEGF-related protein, placenta growth factor (PlGF), discovered in 1991, owes its name to the predominance in placental tissue. It was later identified as a member of the VEGF family as the molecule shares 53% of a homologous domain with the platelet derived growth factor-like region of VEGF.[100] Three isoforms arise by means of alternate splicing, PlGF-1/PlGF131, PlGF-2/PlGF152 and PlGF-3.[101] These molecules are, like VEGF, dimeric glycoproteins. However, the PlGF expression pattern is limited to the placenta and some forms of tumours such as brain tumours and renal cell carcinoma.[102,103] PlGF homodimers bind VEGFR-1 (Flt-1), but have little effect on angiogenesis in vitro.[101] On the other hand, naturally occurring VEGF/PlGF heterodimers, identified in rat glioma cells, are mitogenic; their potency is approximately sevenfold lower than that of the VEGF homodimer. Taking into consideration differential binding affinity and reports of hypoxia-induced up-regulation of VEGF/PlGF in vitro, it seems possible that PlGF and VEGF may be coexpressed in vivo.[102–104]

Angiopoietin

A further family of growth factors involved in the early processes of angiogenesis and vasculogenesis are the angiopoietins. One isotype, angiopoietin 1 (Ang1) is present in tissues adjacent to blood vessels suggesting a paracrine mode of action, whilst another, angiopoietin 2 (Ang2) is only found at sites of tissue remodeling.[105,106] Both angiopoietins, including the two recently discovered angiopoietin-3 (in mouse) and angiopoietin-4 (in humans), have been identified as ligands for the Tie-2/Tek receptor.[105,107] In vitro neither Ang1 nor Ang2 have mitogenic effects mediated via Tie-2.[105] However, Ang1 facilitates endothelial cell sprouting and vascular network maturation.[58,108] Ang2 antagonises Ang1 by blocking Ang1-induced phosphorylization of Tie-2.[106] On the other hand Ang2, in combination with VEGF, promotes neovascularization.[58] Knockout mice for either Tie-2 or Ang1 genes demonstrate an embryonic lethal phenotype caused by defective embryonic development of the vasculature resulting in immature vessels and lack of branch network.[109,110] The findings indicate a contribution of the angiopoietin/Tie-2 system at later stages in the vascular development. This system appears to be particularly involved in the determination of the subdivision of the initially homogeneous capillary network into larger arterioles and venules.[110] A mutation of the RTK Tie-2 in mice leads to vascular dysmorphogenesis, possibly instigated by a lack of peri-endothelial support cell recruitment resulting in underdevelopment of smooth muscle cell layers.[111]

VEGF receptors

In humans, the effects of VEGF on endothelial cells is mediated via two high-affinity membrane-spanning receptors, VEGFR-1 and VEGFR-2. They

Table 4 VEGF receptors

Receptor	Ligand	Function
VEGFR-1 (Flt-1)	VEGF-A_{121} VEGF-A_{165} VEGF-B PlGF-1 PlGF-2	Promotion of cell migration Organization of blood vessels Gene expression of monocytes and macrophages
VEGFR-2 (KDR, Flk-1)	VEGF-A_{121} VEGF-A_{145} VEGF-A_{165} VEGF-C VEGF-D	Mitogenesis, differentiation of endothelial cells Promotion of cell migration Enhancement of vascular permeability
VEGFR-3 (Flt-4)	VEGF-A_{145} VEGF-A_{165} VEGF-A_{189} PlGF-2 VEGF-B_{167}	Remodelling of primary capillary vasculature Embryonic cardiovascular development Regulation of growth and maintenance of lymphatic system
Neu-1	VEGF-A_{165} PlGF-2	Development of cardiovascular system
Neu-2	VEGF-A_{165}	Organization of peripheral nerve fibres Development of vascular networks

are also referred to as RTK. Both receptors have a high affinity for VEGF and possess seven characteristic immunoglobulin-like domains that form the extra-cellular section. Additionally, a kinase-insert domain links a single transmembrane region and a consensus tyrosine kinase.[112–115] VEGFR-1 and VEGFR-2 are 33% identical in their extra-cellular domain and 80% in their kinase domains. Both receptors are predominantly expressed on endothelial cells, but have also been detected on human uterine, colonic and aortic smooth muscle cells, trophoblasts and in foetal kidney.[116,117] VEGFR-3 is a further RTK with seven immunoglobulin-like domains. This receptor is mainly expressed in lymphatic vessels and binds only VEGF-C and -D[118] (Table 4).

VEGFR-1

Vascular endothelial growth factor receptor-1 (VEGFR-1) also known as fms-like tyrosine kinase-1 (Flt-1), is a 180 kDa surface associated RTK.[115] The human gene is located on chromosome 13q12.[119] Flt-1 and VEGFR-2 are predominantly expressed on the vascular endothelium, but traces of mRNA have been located in monocytes, renal mesangial cells and stroma of human placenta.[120–122] PlGF, VEGF-A_{121}, VEGF-A_{165}, and VEGF-B, associate with this receptor with varying affinity.[123,124] VEGF-A_{165} binds to VEGFR-1 with high affinity than VEGF-A_{121}.[125,126] The ability of the receptor to attach heparan-sulphate proteoglycan is eluded after the removal of the second immunoglobulin-like domain of VEGFR-1.[127]

In addition to the full-length receptor, the VEGFR-1 gene encodes for a soluble form carrying only six immunoglobulin domains. This form results from differential splicing of the Flt-1 mRNA and was first discovered in human umbilical vein endothelial cells.[128,129] This soluble receptor, referred to as soluble Flt-1 (sFlt-1), attaches itself to $VEGF_{121}$ with a high affinity, and is present in human plasma[15,77] and amniotic fluids from pregnant women.[130,131] Currently, the biological implications of sFlt-1 remain unknown although in vitro studies have demonstrated that it is capable of reducing VEGF-induced mitogenesis.[128,129] Therefore, sFlt-1 may correspond to a physiological regulatory mechanism for reducing VEGF action.

VEGFR-2

The gene of the second VEGF tyrosine-kinase receptor, VEGFR-2, is located on chromosome 4q12.[132] VEGFR-2 is also known as kinase-insert-domain containing receptor (KDR), and is homologous to the foetal liver kinase-1 (flk-1) receptor in mice. KDR is predominantly expressed in endothelial cells and was cloned from a human endothelial cell cDNA library.[133–135] However, the mRNA for this receptor can also be detected in haematopoietic stem cells, megakaryocytes and retinal progenitor cells.[136–140] VEGFR-1 and VEGFR-2 transduce signals for endothelial cells in response to ligands of the VEGF family. Their individual reaction is distinctively different. Unlike Flt-1, the final glycosylated form of KDR undergoes VEGF-triggered autophosphorylation, which may explain the much

weaker response to VEGFR-1 activation.[141] KDR binds $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ VEGF-C and VEGF-D.[126,142] Despite numerous similarities between VEGFR-1 and VEGFR-2, a naturally occurring soluble form of KDR comparable to sFlt-1 has not been described.

VEGFR-3

The VEGFR-3 gene is encoded in the chromosomal region 5q34–q35.[143] VEGFR-3 is also known as fms insert-like tyrosine kinase 4 (Flt-4) and its extracellular domain is 80% homologue to the other VEGFR.[118] Only VEGF-C and VEGF-D of the VEGF family are associated with Flt-4.[83,93] Unlike VEGFR-1 and VEGFR-2, Flt-4 is predominantly expressed in lymphatic endothelium in adult tissue.[85,95,144] However, in most vascular endothelial cells low levels of VEGFR-3 are detectable. Its presence, particularly on lymphatic endothelial cells and on developing vessels of several organs suggests that Flt-4 together with its ligands may have a role in the regulation of growth and differentiation of the lymphatic system.[96]

Neuropilins

In addition to VEGFR-1 and VEGFR-2, endothelial cells express neuropilin-1 (Neu-1) and neuropilin-2 (Neu-2), which selectively bind (but with low affinity) VEGF-A_{165}. Due to a short intracellular domain of these receptors they are not likely to operate as an independent receptor. This is further supported by lack of cellular response when stimulating only the neuropilins.[145] However, during the embryonic stages of angiogenesis neuropilin-1 seems to regulate blood vessel development, suggesting a role as coreceptor for VEGFR-2.[146] The genetic encoding and exact biological purpose has yet to be discovered.

Regulation of VEGF production

As a key regulator, it is essential that the expression of VEGF is itself correctly controlled in order to prevent uncontrolled angiogenesis. There are a plethora of cytokines, growths factors and physiological parameters modulating the production of VEGF, depending on the current status quo. In the mature organism, VEGF expression is limited and a balance between angiogenic and anti-angiogenic stimuli is maintained.[41] However, in response to tissue damage, a wide array of growth factors, cytokines and other molecules is released stimulating angiogenesis directly or indirectly via VEGF which is essential for the repair process.

In pathophysiological situations such as cancer and diabetes mellitus, stimulated VEGF expression might result in increased pathological angiogenesis. This hypothesis is further supported by data demonstrating a suppression of neovascularization by inhibition of VEGF or its effects.[147,148] However, in other circumstances, such as atherosclerosis and diabetes, the increased plasma VEGF concentration[77] might be an attempt to compensate for tissue damage or hypoxia, or may simply reflect endothelial cell damage apparent in these conditions.

The interaction of VEGF with cytokines and other growth factors

Factors that can alter VEGF production include platelet derived growth factor, tumour necrosis factor-α (TNF-α), fibroblast growth factor 4 (FGF 4), bFGF, transforming growth factor-β (TGF-β), PDGF, angiotensin-2, insulin-like growth factor I, keratinocyte growth factor, interleukin 1 (IL-1) and IL-6.[69,149–160] A few substances, such as the cytokines IL-10 and IL-13, decrease VEGF production.[161]

The angiopoietins also influence VEGF release.[85,105] Ang-1 stimulates vessel sprouting whereas Ang-2 inhibits this effect, but also mediates destabilization of vessel integrity, which in turn facilities vessel sprouting in response to VEGF.[106,110,162] These effects are mediated via the Tie-2 receptor. The combination of VEGF, Ang-1 and Ang-2 is essential for successful angiogenesis as established in vivo experiments.[58]

Effect of oxygen on VEGF expression

Apart from growth factors there is a variety of chemical stimuli affecting the release of VEGF. Hypoxia, which occurs in pathophysiological processes such as atherosclerosis, solid tumours and proliferative retinopathy, is a major stimulator of VEGF expression resulting in neovascularization.[163] Hypoxia induces a protein called hypoxia inducible protein complex (HIPC) or hypoxia-inducible factor (HIF).

This heteromeric basic helix–loop–helix transcriptional regulator is activated by reduced oxygen tension and up-regulates the transcription of VEGF mRNA. HIF increases production of VEGF mRNA with enhanced stability by directly attaching to a HIF-1 binding-site located in the VEGF promoter region.[67,164,165] Furthermore VEGFR-1 seems to be up-regulated through hypoxia induced HIF.[166]

Hypoxia not only increases VEGF production but it also seems to increase the stability of some VEGF isoforms.[149,167–169] With regard to stability, VEGF-A

isoforms are hypoxia sensitive whereas hypoxia has little or no effect on VEGF-B and VEGF-C mRNA.[66] This variation in the behaviour of VEGF isoforms may be another regulatory mechanism, that ensures that the different VEGF species are tissue and/or functionally specific.

Further mechanisms leading to hypoxia-induced increase of VEGF production may be related to often associated features of hypoxia such as tissue damage, necrosis and apoptosis. These events may therefore trigger the release of cytokines and other chemical mediators from cells of the surrounding tissue, initiating a cascade of events leading to the production of VEGF.[65,170] These events are discussed below.

The importance of oxygen as a regulator of VEGF production is further emphasized by demonstrating inhibitory properties of the normoxic or even hyperoxic environment. Hypoxia-induced VEGF increase returns to baseline levels within 24 h of the return of the cells to normoxia.[171] VEGF expression is decreased in in vitro and in vivo studies following hyperoxia.[172,173] Additionally, hyperoxia-induced retinopathy in prematurely born mice can be prevented by intraoccular VEGF injection.[174] These data clearly demonstrate the importance of oxygen as a regulatory mechanism of VEGF expression.

Regulation of VEGF by nitric oxide

VEGF is known to induce the release of nitric oxide (NO) from endothelial cells, and vascular endothelium and inducible NO synthase (iNOS) production is amplified during VEGF-induced angiogenesis. Therefore the physiological effects of VEGF may, at least in part, be mediated by endothelium derived NO.[175,176] The vital role of NO in VEGF-induced angiogenesis has also been demonstrated in NOS knock-out mice as well as after NOS inhibition, both resulting in reduction of angiogenesis.[175,177] NO, on the other hand, also has regulatory effects on VEGF production. Protein kinase C mediated binding of the transcription activator protein-1 (AP-1) is decreased by NO.[178] This results in reduced stimulation of the promoter region of the VEGF gene, hence lower VEGF expression. Pathological circumstances coupled with impaired NO availability, such as atherosclerosis, are associated with increased VEGF levels consistent with the presence of a negative feedback loop.[178,179] Increased levels of plasma VEGF have been demonstrated in patients with various risk factors for atherosclerosis such as diabetes mellitus and hypertension,[15,77] further supporting this theory although, as discussed, raised VEGF may also be related to tissue hypoxia or may simply reflect endothelial damage. The same rationale may also partly explain raised plasma VEGF in certain cancers[180,181] as the demands of the growing tumour may create a local hypoxia.

Effect of glucose on VEGF expression

Hypoglycaemia increases VEGF expression, which was initially thought to be an indirect consequence mediated via associated hypoxia. However, up-regulation and increased production of VEGF have been described in cells exposed to hypoglycaemia independently of HIF (hypoxia).[169,182–184] After equilibration of the glucose concentrations VEGF production returned to pre-experimental levels[184] suggesting that acute hypoglycaemia may trigger VEGF mediated angiogenesis.

Furthermore,[185] increased intracellular Ca^{2+} levels in a glucose-deprived environment leads to activation of protein kinase C. This process induces the activation of AP-1 resulting in increase of VEGF expression, thus not only confirming previous studies but exposing its underlying mechanism.

Remarkably, not only lack of glucose but also high glucose levels result in an upsurge of VEGF mRNA,[150,186,187] as well as production of VEGF and VEGFR-2.[180] Recent studies have demonstrated that hyperglycaemia can directly increase VEGF expression via a protein kinase C dependent mechanism, and this effect can be abolished by a protein kinase C inhibitor.[186–188] Hyperglycaemia induced VEGF up-regulation is also reversible by normalizing the extra-cellular glucose concentration in SMC.[150] Therefore, and possibly difficult to explain simply, and type of non-euglycaemia seems a strong up-regulatory factor for VEGF expression. Hence the apparent relationship between angiogenesis, VEGF and diabetes[4,77,137,189] requires clarification.

Pathophysiological consequences of the interactions between growth factors and their receptors

The importance of the specific angiogenic activities of VEGF and its receptor interactions in the process of endothelial cell proliferation, differentiation, migration and growth has been considerably enhanced by analysis of knock-out mice.[190,191] The pattern of abnormalities observed provides some evidence for the role of VEGF and its receptors Flt-1, KDR and Flt-4, along with Tie-2/Tek and its ligands angiopoietins 1 and 2. Certainly, all four receptors are essential for vasculogenesis as mutations in the loci of any of the gene coding for these

receptors leads to embryonic lethality due to imperfections in the haemotopoietic and endothelial cell lineage. Mutations in different genes encoding VEGF or its receptors become evident as different phenotypic defects.[192,193] Homozygous VEGF receptor deficiency resulting in embryonic death varies from heterozygous VEGF gene mutation, which generates an embryonic lethal phenotype.[192,193]

There are also different patterns arising from receptor mutants. Unlike KDR, Flt-1 not only affects endothelial cell proliferation and differentiation, but also blood vessel construction as demonstrated by certain mutations in Flt-1 loci causing embryonic lethality due to inadequate vessel assembly.[19,42] After targeted inactivation of the Flt-4 gene, vasculogenesis and angiogenesis occur but the large blood vessel development is disorganized with irregular sized vessels and defective lumens leading to cardiovascular failure.[162] However, mutation in the genes for angiopoietin or its receptors results in disrupted vessel structure and impaired capillary functions leading to haemorrhage.[106,110] Findings from these studies suggest that in embryonic vasculogenesis, KDR-mediated processes precede those of Flt-1. KDR is involved in endothelial cell formation, proliferation and migration in the early stages of vasculogenesis, whilst Flt-1 plays a role in embryonic vascular assembly following differentiation of endothelial cells. At an even later stage Flt-4 is involved in organizing large vessels and the emergence of lymphatic vessel formation but preceding the angiopoietins and their receptors.[106,110] Table 2 details the phenotypic mutations observed with targeted gene mutation of VEGF-A, the angiopoietins and their respective receptors.

Summary and clinical perspectives

The majority of our knowledge of VEGF originates from work done as part of studies in cancer research, as the ability of a tumour to metastasize seems be related to the quantity of VEGF produced.[134] VEGF has been detected in numerous tumour cells and in the plasma of patients with various cancers,[101,180,181,194–202] and hypoxia appears to play an important part as the expression of VEGF mRNA and production of the growth factor is intensified in regions neighbouring the necrotic area.[197,203] Furthermore, surgical excision of a localized tumour resulted in a prompted reduction in circulating VEGF.[204] In addition, VEGF may also have a role in the regulation of inflammatory repair processes as VEGF increases vascular permeability and acts as chemotactic agent for phagocytic cells, both processes of eminent importance during inflammation.[205] VEGF expression is dramatically up-regulated in chronic wounds such as venous leg ulceration particularly in the hyperplastic epithelial region of the wound margin.[206] Similar findings have been observed in resected liver where higher levels of VEGF have been demonstrated when compared to normal liver.[207] Again hypoxia, a common feature in damaged tissue, seems to be the underlying mechanism.[208]

In chronic inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus, raised levels of VEGF have been noted in plasma, serum and synovial fluid.[209,210] Regrettably, however, in some of these cases (and, indeed, in any clinical study), VEGF data derived from serum is of limited value in the study of pure vascular responses as VEGF may also arise from platelets.[80,211] However, the existence of VEGF in the sub-synovial macrophages, leukocytes, fibroblasts and synovial lining cells implies some participation in the inflammatory process.[212,213] Indeed, it has been suggested that the amount of VEGF in rheumatoid synovium may be a marker for joint destruction.[214] Overall, therefore, it appears plausible that VEGF-induced angiogenesis and increased vascular permeability may promote these chronic inflammatory processes. More recently, possible roles for VEGFs C and D and their receptors in the development of arthritic synovia have been proposed.[215]

Recently, a link between VEGF and cardiovascular disease has been established. Atherosclerosis eventually results in progressive arterial occlusion which leads to ischaemia, hypoxia and subsequently to necrosis. These processes trigger the expression of a variety of vasoactive substances, matrix proteins and growth factors, which mediate neovascularization, remodelling of the vasculature and surrounding tissue.[203] Animal studies of VEGF in various aspects of cardiovascular disease[216–220] have provided pilot data for studies in man. For example, histological studies of coronary atherosclerotic plaques, saphenous vein bypass grafts, and areas of recent myocardial infarction that demonstrated increased VEGF expression[221–224] have given way to observational clinical studies.[225,226]

Pathophysiological, possibilities include the suggestion that acute myocardial ischaemia rapidly induced up-regulation of VEGF and its receptors VEGFR-1 and VEGFR-2, whereas areas of healed myocardial infarction failed to demonstrate that effect.[216,220] These data would suggest that VEGF plays a role in neovascularization in connection with myocardial ischaemia and atherosclerotic arteries. Atherosclerotic lesions in human coronary arteries demonstrate distinct expression of VEGF,

Table 5 Human tissue/cell studies of VEGF and angiogenesis in cardiovascular disease

References	Study sample(s)	Observations	Comments
Couffinhal et al., 1997[223]	Normal arteries, veins and atherosclerotic coronary arteries	VEGF was immunolocalized predominantly to SMC in normal and atherosclerotic vascular tissue	Localization of VEGF to normal and atherosclerotic vascular tissue implicates VEGF in vascular physiology
Ruef et al., 1997[218]	Umbilical vein endothelial cells and vascular smooth muscle cells	VEGF expression of vascular endothelial cells increased after oxidative stress and balloon-injuries	VEGF may enhance neovascularization of atherosclerotic and restenotic arteries
Inoue et al., 1998[221]	Coronary artery segments stained for VEGF, VEGFR-1 and VEGFR-2	VEGF activity, VEGFR-1 and VEGFR-2 were detected in atherosclerotic but not normal arteries	Small autopsy study, role for VEGF in progression of CAD as well as recanalization
Chen et al., 1999[222]	Coronary artery	Number of VEGF positive cells correlates with number of intimal blood vessels	More advanced type of atherosclerotic lesion contain more VEGF positive cells
Lee et al., 2000[220]	Biopsied myocardial tissue	Expression of VEGF mRNA was more pronounced in tissue with acute ischemia compared with normal ventricle or those with past episodes of infarction	Acute myocardial injury may result in increased production of VEGF transcripts in humans
Bobryshev et al., 2001[224]	Aortocoronary saphenous vein grafts	Areas of intimal neovascularization and neovascular endothelial cells were VEGF positive	VEGF local regulator of intimal neovascularization in saphenous vein grafts

Table 6 Summary of studies measuring VEGF in the plasma or serum of patients with cardiovascular disease

References	*n*	Observations	Comments
Seko et al., 1997[78]	19	Serum VEGF levels were significantly elevated in patients with AMI compared to controls. After reperfusion, levels were normalized	Small observational study, illustrating the acute induction of circulating VEGF and also reflecting the relatively short half-life of this growth factor
Fleisch et al., 1999[225]	76	Levels of intra-coronary VEGF in patients undergoing angioplasty correlated with collateral flow and proximal VEGF levels were higher in patients with more stenotic lesions	Large observational study suggesting that serum VEGF levels may be dependent on the degree of coronary atherosclerosis and/or disease severity
Hojo et al., 2000[227]	30	Levels of VEGF in serum increased gradually after the onset of AMI and peaked on day 14	Medium sized progressive study, also showing acute induction of VEGF in humans with CVD
Burton et al., 2000[226]	32	Post-operative serum VEGF levels were significantly greater than pre-operative levels following coronary artery bypass surgery	Medium sized comparative study showing acute induction of VEGF following cardiovascular surgery
Belgore et al., 2001[15]	21	Levels of plasma VEGF and sFlt-1 were significantly raised in hypertensives compared with controls and these were normalized after successful therapy	Medium sized intervention study highlighting the possible involvement of VEGF in hypertension and the effect of therapy on levels of VEGF and sFlt-1
Blann et al., 2002[77]	140	Levels of plasma VEGF were significantly raised while levels of sFlt-1 were lower in the patients with PAD or CAD compared with controls. Also, diabetic data.	Large comparative study of two patient groups with variant atherosclerosis. First study to examine the levels of sFlt-1 in plasma in atherosclerosis.

n, number of patients.

VEGFR-1 and VEGFR-2 on endothelial cells, macrophages and partially differentiated smooth muscle cells.[221,222] Moreover, in patients with coronary artery disease there is a correlation between the directly measured index of collateral blood flow and intracoronary levels of VEGF, suggesting that VEGF is influenced by degree of coronary atherosclerosis.[225] However, generally, the precise role(s)

of large amounts of circulating VEGF in the plasma of subjects with long-standing peripheral or coronary atherosclerosis, or in acute myocardial infarction compared to asymptomatic controls[77,78,227,228] is unclear. As histological data confirms amplified angiogenicity in atherosclerotic lesions by demonstrating a plethora of blood vessels within the atheromatous plaques itself and in the surrounding vessel walls,[221–223,229,230] VEGF-mediated neovascularization of the media and adventitia of diseased vessels may be relevant in enhancing the supply of oxygen and nutrients to the affected tissue.[231]

Against this background is the presumption by many commentators that exogenous VEGF supplied as a therapy may provide a benefit in cardiovascular disease by enhancing collateral development[232–235] and preliminary methodological work has been published[236–238] with some success.[239] However, recent animal data suggest that exogenously-supplied VEGF may actually enhance atherosclerotic plaque progression,[240] implying that raised plasma VEGF in man[77,78,227,228] may not be advantageous. Indeed, rheumatologists, studying a different disease where there is raised plasma VEGF[209,210] and evidence of involvement in pathogenesis,[212–215] seek to reduce angiogenesis,[241] as do oncologists.[40,181,194,196,204,242]

The involvement of VEGF in atherosclerosis therefore seems undoubted, as summarized in Tables 5 and 6 although its precise effects (and the value of interventions) are subject of an ongoing debate. It is heartening to note from recent data that therapeutic angiogenesis (e.g. with recombinant fibroblastic growth factor-2) in intermittent claudication *does* provide some clinical benefit, at least in phase II trials.[243] Nonetheless, the variable results in clinical trials could at least in part reflect the inadequacy of preclinical in vitro and animal models. Only time will tell whether this approach would bring the potential morbidity and mortality benefits that we hope would arise.

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